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THE INDUCTION OF D-XYLOSE CATABOLIZING ENZYMES IN PACHYSOLEN TANNOPHILUS AND THE RELATIONSHIP TO ANAEROBIC D-XYLOSE FERMENTATION

Magdalena E. Ligthelm, Bernard A. Prior* and James C. du Preez

Department of Microbiology, University of the Orange Free State, P.O. Box 339, Bloemfontein 9300, South Africa

Summary

Aerobic cultures harvested from the lag and early exponential growth phases fermented D-xylose poorly under anaerobic conditions whereas fermentation by late exponential and stationary phase cultures was rapid. These differences could be related to the ratios of NADH- to NADPH-linked xylose reductase (XR) and the levels of NADH-linked XR and NAD-linked xylitol dehydrogenase (XD) present. Under aerobic conditions, induction of NADPH-linked XR preceded NADH-linked XR which suggested the presence of two separate XR's. Induction of XR and XD was more rapid under aerobic than anaerobic conditions.

Introduction

Discrepancies exist with regard to the ability of *Pachysolen tannophilus* to ferment D-xylose anaerobically. Significant differences in the maximum specific ethanol production rate ($q_p max$) and maximum ethanol concentration produced were reported by different researchers (Schneider et al. 1981, 1983; Jeffries 1982; Slininger et al. 1982a,b; Schvester et al. 1983; Suihko and Drazic 1983; Bruinenberg et al. 1984; Du Preez et al. 1984; Watson et al. 1984) which could be related to the phase of aerobic growth where the inocula were harvested. Furthermore, Bruinenberg and coworkers (1983, 1984) ascribed the slow anaerobic fermentation of D-xylose by *P. tannophilus* to a low NADH-linked xylose reductase (XR) activity whereas rapid anaerobic fermentation of D-xylose by *Pichia stipitis* was related to a high NADH-linked XR activity.

In order to clarify these discrepancies, the activity of the two enzymes catalyzing the initial steps of D-xylose metabolism, namely xylose reductase (XR, EC 1.1.1.21) and xylitol dehydrogenase (XD, EC 1.1.1.14), in relation to various cofactors (NADPH, NADH and NADP, NAD respectively) was determined at various growth phases of the culture. In addition, the

relationship between enzyme activities and the anaerobic fermentation of D-xylose was investigated.

Materials and Methods

Microorganism and culture medium. The origin, maintenance and medium for the cultivation of *Pachsolen tannophilus* NRRL Y-2460 were described previously (Ligthelm et al. 1988).

Aerobic growth experiments. The inoculum was prepared by aerobic cultivation of the organism at 30°C in a 1 ℓ Erlenmeyer flask containing 100 mℓ D-glucose (40 $g.\ell^{-1}$) culture medium for 24 h on an orbital shaker (160 $rev.min^{-1}$; 27.5 mm throw). The cells were harvested by centrifugation (2460 x g for 5 min), washed once with sterile water and used to inoculate 1 ℓ Erlenmeyer flasks equipped with side-arm cuvettes and containing 100 mℓ D-xylose (40 $g.\ell^{-1}$) culture medium. Growth was measured by turbidimetry (A_{640}) and at certain stages during the exponential and stationary phases of growth cells from flasks were harvested, washed once and used for anaerobic fermentation experiments or held frozen (-70°C) for enzyme assays.

Anaerobic fermentation experiments. Cells were injected into 30 $m\ell^{-1}$ serum vials to an initial cell density of 0.7 to 1.7 g dry biomass.ℓ^{-1}. The contents of the vials were gassed with scrubbed nitrogen (Hungate 1969) for 15 min before and after inoculation. These vials (filled almost completely and tightly sealed) were cultivated at 30°C on an orbital shaker (150 $rev.min^{-1}$; 20 mm throw).

Preparation of cell extracts. Cells were washed twice with pH 7.5 buffer {10 mM K_2HPO_4; 0.5 mM ethylene diamine tetracetic acid; 0.5 mM 2-mercaptoethanol (Ditzelmüller et al. 1984b)}. The cells were disrupted with glass beads (0.5 - 1.0 mm) by vortexing for five 1 min bursts with 1 min intervals of cooling on ice. Cell debris was removed by centrifugation (28,000 x g at 4°C for 20 min).

Analytical methods. Concentrations of biomass, ethanol, D-xylose and xylitol were determined as described previously (Ligthelm et al. 1988). The xylose reductase assay reaction mixture contained: 1.0 M K_2HPO_4 (pH 7.4), 0.5 mℓ; 0.1 M 2-mercaptoethanol, 0.1 mℓ; 0.5 M D-xylose, 0.1 mℓ; 3.4 mM NADPH or NADH, 0.1 mℓ; cell extract and water to 1 mℓ. The reaction was started by the addition of D-xylose and the A_{340} decrease was measured (Smiley and Bolen, 1982). The xylitol dehydrogenase assay reaction mixture contained: 0.5 M Tris (pH 8.6), 0.15 mℓ; 0.5 M xylitol, 0.1 mℓ; 3.4 mM NAD or NADP, 0.1 mℓ; 5 mM $MgCl_2$, 0.05 mℓ; cell extract and water to 1 mℓ. The A_{340} increase was measured after xylitol addition (Smiley and Bolen 1982; Bruinenberg et al. 1983b). Blanks containing all the components except the substrate were used to correct for endogenous enzyme activity which was minimal. In all assays the reaction velocity was linearly proportional to the amount of extract present over an 8 min period. The protein concentration of cell extracts was measured using bovine serum albumin as standard (Lowry et al. 1951). One unit (U) of activity was defined as the amount of enzyme which catalyzed the oxidation of 1 nmol NADPH or NADH per min or the reduction of 1 nmol NAD or NADP per min at 30°C.

Results and discussion

When D-glucose-grown *P. tannophilus* was transferred to a D-xylose-containing medium and cultivated under aerobic conditions, an initial lag of 8 h was followed by rapid growth and the stationary phase was attained after

30 h (data not shown). Cells from the lag and early exponential growth phases (0 h and 9 h old) produced little or no ethanol when transferred to anaerobic conditions. However, cells from the late exponential or early stationary growth phases (16 h and 32 h old) resulted in a more rapid ethanol production with the specific rate of ethanol production reaching a maximum in the initial stages of fermentation (Fig. 1). Under anaerobic conditions, the culture either failed to grow or growth was restricted to less than one doubling. Poor growth during anaerobic D-xylose fermentation has been reported previously but without explanation (Jeffries, 1982; Slininger et al., 1982a; Bruinenberg et al., 1984; Du Preez et al., 1984; Neirinck et al., 1984).

The importance of the cofactor balance during anaerobic fermentation of D-xylose by *P. tannophilus* was initially pointed out by Bruinenberg et al. (1984) and this led us to investigate the induction of XR and XD in order to explain the results obtained in Fig. 1. During aerobic growth, the specific activity of the NADPH-linked XR increased more rapidly and at an earlier stage than the NADH-linked enzyme (Fig. 2). This is reflected by the increase in the ratio (NADH/NADPH) of the specific activities of XR that reached a constant value ($\sim$0.3) during the late exponential and stationary growth phases. An increase in the specific activity of the NAD- and NADP-linked XD commenced simultaneously (Fig. 3), with a linear increase in the ratio of NAD- to NADP-linked activity. The NAD-linked XD specific activity was considerably greater than the NADP-linked activity at all growth phases and suggests that the NAD-linked enzyme is more important in the utilization of D-xylose. Furthermore, the NADP-linked activity may reflect affinity for the XR (reverse direction) present in the extract rather than actual XD activity. Other workers found a similar low activity of NADP-linked XD in *P. tannophilus* (Bruinenberg et al., 1984; Ditzelmüller et al., 1984a; Morimoto et al., 1986).

Under anaerobic conditions, increases in the specific activities of NADPH- and NADH-linked XR were observed but induction occurred at a much slower rate than found under aerobic conditions and the levels of XR attained in the culture from the 9 h old inoculum were lower than found in cultures from older inocula (20 h and 32 h; Table 1). The specific activities of NAD-linked but not the NADP-linked XD increased during anaerobic fermentation and the increase was more rapid in cultures containing older cells (Table 1).

The fermentative ability of the aerobically grown culture appeared to be dependent upon the initial levels of NADH-linked XR present as the rate of anaerobic fermentation was slower in cultures inoculated with 9 h old cells

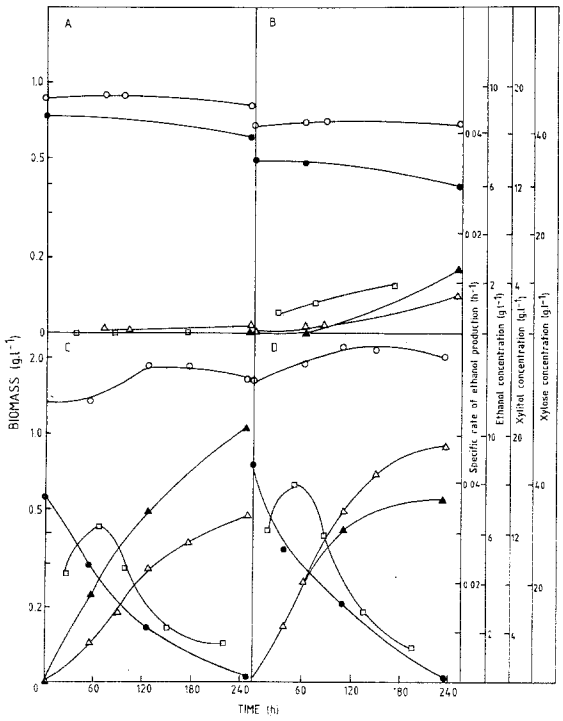

Fig. 1. Relationship of D-xylose (40 g.ℓ^{-1}) fermentation by *Pachysolen tannophilus* under anaerobic conditions to inoculum age (A, 0 h; B, 9 h; C, 16 h; D, 32 h) grown under aerobic conditions. Specific rate of ethanol production (q_p max, □), concentrations of ethanol (△), xylitol (▲), biomass (○) and D-xylose (●).

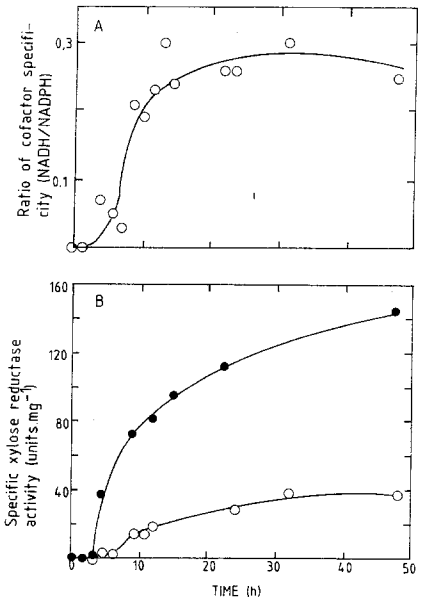

Fig. 2. The induction under aerobic conditions of xylose reductase in *Pachysolen tannophilus* after transfer to D-xylose-containing medium. A: the ratio of cofactor specificity (NADH/NADPH). B: the specific NADPH (●)- and NADH (○)-linked xylose reductase activity.

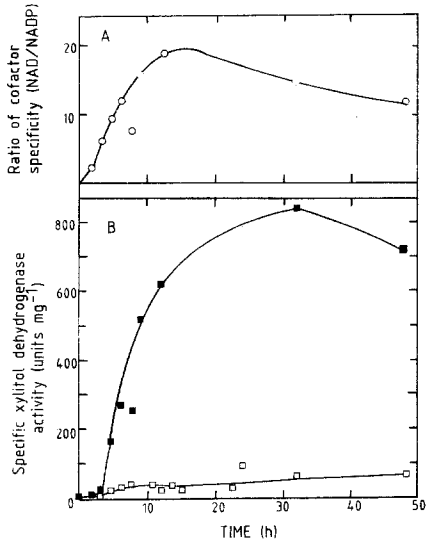

Fig. 3. The induction of xylitol dehydrogenase under aerobic conditions in *Pachysolen tannophilus* after transfer to D-xylose-containing medium. A: the ratio of cofactor specificity (NAD/NADP). B: the specific NAD (■)- and NADP (□)-linked xylitol dehydrogenase activity.

TABLE 1

Ethanol and enzyme production under anaerobic conditions by *Pachysolen tannophilus* in a medium containing 40 $g.\ell^{-1}$ D-xylose[a]

Inoculum age (h)	Time under anaerobic conditions (h)	Maximum ethanol concn ($g.\ell^{-1}$)	Specific ethanol productivity (h^{-1})	Enzyme activities ($U.mg^{-1}$)[b]					
				Xylose reductase			Xylitol dehydrogenase		
				NADPH-linked	NADH-linked	Ratio NADH/NADPH	NAD-linked	NADP-linked	Ratio NAD/NADP
9	0	0	-	27(2)	7(0)	0.22	370(26)	17(1)	22
	114	0.3	0.004	10(3)	7(0)	0.60	98(29)	2(0)	49
	249	2.1	0.03	63(1)	35(1)	0.56	433(139)	22(1)	18
20	0	0	-	72(7)	27(2)	0.38	472(14)	30(1)	16
	104	7.0	0.05	66(1)	36(2)	0.55	357(131)	14(1)	26
	239	9.3	0.02	258(56)	61(7)	0.24	1046(102)	18(1)	61
32	0	0	-	54(7)	25(2)	0.46	267(33)	24(0)	11
	92	4.9	0.07	64(2)	32(2)	0.50	287(40)	12(1)	24
	228	8.2	0.03	116(20)	58(4)	0.50	633(103)	24(1)	29

[a]The inoculum was prepared on D-glucose under aerobic conditions.

[b]Mean of two determinations with the standard error of the mean indicated in brackets.

than fermentation with older cells containing higher initial levels of NADH-linked XR (Table 1). NADH-linked XR is required to recycle NADH generated by the reduction of xylitol to xylulose (Bruinenberg et al., 1984) as the anaerobic conditions preclude regeneration through mitochondrial respiration. Transhydrogenase activity is absent in yeasts (Van Dijken and Scheffers, 1986) and thus accumulated NADH cannot reduce NADP produced in the XR (D-xylose → xylitol)-reaction.

The differential induction of NADPH- and NADH-linked XR activity and its effect on anaerobic D-xylose fermentation is consistent with the multiple forms of XR in *P. tannophilus* reported by Verduyn et al. (1985). They isolated two enzymes: one with dual NADPH-/NADH-linked activity (also found by Bolen et al., 1986) and the other with an activity linked only to NADPH similar to that purified by Ditzelmüller et al. (1984b). Ditzelmüller and co-workers (1985) also isolated a second XR but with NADH-linked activity only. These apparent contradictory results could be explained by the fact that growth conditions determined the NADH- to NADPH-linked ratio since growth under oxygen limitation increased this ratio significantly (Verduyn et al., 1985). Our data also suggest that oxygen levels might affect the production of NADPH- and NADH-linked XR because the dissolved oxygen tension would decrease during aerobic cell growth as cell density (and age) of the culture increased. Alternative explanations for these results may

exist. Jeffries (1985) has suggested that composition and pH of the growth medium may affect the rate of anaerobic fermentation.

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